(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 March 2003 (13.03.2003)

PCT

(10) International Publication Number
WO 03/020111 A2

(51) International Patent Classification[7]: A61B

(21) International Application Number: PCT/US02/27533

(22) International Filing Date: 29 August 2002 (29.08.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/316,244 30 August 2001 (30.08.2001) US

(71) Applicant: **MOUNT SINAI SCHOOL OF MEDICINE OF NEW YORK UNIVERSITY** [US/US]; One Gustave L. Levy Place, New York, NY 10029-6574 (US).

(72) Inventors: **NEMERSON, Yale**; 145 Nassau Street, Apartment 10C, New York, NY 10038 (US). **BOGDANOV, Vladimir**; 508 East 79th Street, Apartment 6A, New York, NY 10021 (US).

(74) Agents: **PABST, Patrea, L.** et al.; Holland & Knight LLP, One Atlantic Center, 1201 West Peachtree Street, N.E., Suite 2000, Atlanta, GA 30309-3400 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/020111 A2

(54) Title: ALTERNATIVELY SPLICED CIRCULATING TISSUE FACTOR

(57) Abstract: A new circulating foml of soluble human tissue factor was identified. This new foml of human tissue factor appears to be the result of alternative splicing and is therefore referred to as "alt-hTF." Alt-hTF mRNA was detected in a cell line, HL-60. The cDNA region encoding the entire open reading frame of alt-hTF was cloned. The sequence encoding the alt-hTF mature peptide was expressed in bacteria. alt-hTF consists of the first 166 amino acids of membrane bound TF, and a 40 amino acid C-temlinal region unique to alt-hTF. Alt-hTF is likely to be a useful target for compounds to inhibit clotting and to treat disorders associated with elevated TF. It may also be useful as a target for antibodies selectively reactive with alt-hTF, to remove it from the circulation for treatment of clotting or other disorders associated with elevated or abnomlallevels of TF, including thrombotic conditions, cardiovascular disorders, DVT, DIC, and possibly metastatic cancers.

clotting assays. The soluble tissue factor has the advantage that it is easier to produce, purify and resuspend, as compared to the membrane bound form.

A shorter form of human tissue factor, consisting of amino acid residues 2-219 (Morrissey et al., Blood 1993; 81: 734-744) or 1-218 (Wildgoose et al., Blood 1992; 80: 25-28), produced by recombinant techniques and expression in bacteria, has been reported to be useful in an assay distinguishing between clotting factor VII and activated factor VIIa, when measured in the presence of high quantities of phospholipid.

In blood vessels of healthy humans, tissue factor is found primarily in the adventitia and thus physically separated from coagulation factors, which mainly circulate in an inactive form. Following injury, TF is exposed to blood and initiates the coagulation cascade. The resulting fibrin formation is essential for the initial repair of vessel damage to minimize blood-loss. Therefore, TF may be considered to form a hemostatic sheath around blood vessels essential for hemostasis and appears to be essential for life inasmuch as no TF deficiency has been reported and TF knockout mice do not survive beyond the perinatal period.

TF also plays a crucial role in pathological situations such as coronary artery disease or deep vein thrombosis (DVT). In the former, atherosclerosis is the underlying process leading to pathological disturbances of the arterial wall. The mechanism of venous thrombosis is poorly understood but perhaps blood-borne TF is involved, as reported by Giesen et al., Proc. Natl. Acad. Sci. USA 1999; 96: 2311-2315. Atheromae contain TF as judged by direct bioassay of excised lesions and by immunohistochemistry. Monocytes/macrophages are generally believed to be the major source of this TF although smooth muscle cells near experimental arterial injury contain TF. Upon plaque rupture, TF is exposed to flowing blood thereby allowing circulating factor VII/VIIa to complex with TF. This complex is the catalyst that initiates blood coagulation and thrombosis. However, the deposition of platelets on a TF-coated disc has been reported to inhibit this surface-bound TF, thus implicating circulating TF as necessary

result of alternative splicing and is therefore referred to as "alt-hTF." alt-hTF mRNA was detected in a cell line, HL-60. The cDNA region encoding the entire open reading frame of alt-hTF was cloned. The sequence encoding the alt-hTF mature peptide was expressed in bacteria. Alt-hTF consists of the first 166 amino acids of membrane bound TF, and a 40 amino acid C-terminal region unique to alt-hTF.

Alt-hTF is used as a diagnostic and is also a target for compounds to inhibit clotting and to treat disorders associated with elevated TF. It is also useful as a target for antibodies selectively reactive with alt-hTF, to remove it from the circulation for treatment of clotting or other disorders associated with elevated or abnormal levels of TF, including thrombotic conditions, cardiovascular disorders, DVT, DIC, and possibly metastatic cancers.

Brief Description of the Drawings

Figure 1 is a schematic of the full-length human tissue factor protein, the alternatively spliced human tissue factor protein, and the regions therein.

Figure 2 is a graph of the TF activity [pM factor Xa/min] for the fractions of human plasma centrifuged at 260,000xG, i.e. starting material (1:1 plasma:TBS), lipid phase (n=3), bulk phase (n=5), viscous liquid (n=3), and pellet (n=1). Tissue factor was captured using immobilized antibodies to TF and then relipidated with 30:70 mixture of phosphatidyl serine and phosphatidyl choline. TF activity was measured by adding calcium (5 mM), clotting factors VIIa (1 nM) and X (150 nM), and measuring the subsequent rate of factor Xa generation.

Figure 3 is a graph of TF activity in human plasma treated with an antibody to alt-hTF.

Figures 4A and 4B are graphs of recombinant alt-hTF activity in two assay systems.

Detailed Description of the Invention

I. Tissue factor compositions

Membrane bound human tissue factor ("TF") is a polymer of 263 amino acids. At one end of the polymer is an amino ("NH_2") group commonly referred to in the art as the amino (or "N")-terminus. At the other

residues at positions 4, 5 and 8 (Phe 4, Leu 5, Leu 8). Studies with coagulation factors IX, X and protein C suggest that occupancy of the Ca^{2+}-binding sites induces the surface exposure of these residues (in IX the residues are at position 6, 9 and 10) which then engage in hydrophobic interactions with the phospholipid layer. Indeed, as seen in the crystal structure of TF:VIIa the side chains of these three conserved hydrophobic residues point away from the rest of the Gla domain in a direction approximately perpendicular to the string of 6 calcium ions. Therefore, in analogy to the proposed function of the homologous residues in other coagulation enzymes, these amino acids may anchor VIIa to membranes by insertion into the outer phospholipid layer.

Mutational changes at TF residues Lys 165 and Lys 166 revealed another region of TF that is important for enzymatic activity of the TF:VIIa complex. These two lysine residues are part of a surface region that interacts with substrates and is located outside the TF-VIIa interface area. The main region is composed of 7 residues (Tyr 157, Lys 159, Ser 163, Gly 164, Lys 165, Lys 166 and Tyr 185) forming a continuous surface patch of about 500 $Å^2$. This substrate recognition region, which may further extend to the VIIa-Gla domain, contacts the Gla domains of substrates X and IX. It may also interact with the EGF-1 domain of substrates as suggested by the impaired activation of two naturally occurring IX-EGF1 domain variants, Gly48Arg and Gly48Val, and by IX-EGF1 domain swap experiments. This TF-substrate contact site may serve to properly align X and IX with respect to TF:VIIa complex allowing the formation of productive ternary TF:VIIa:substrate complexes. In agreement with the assigned functional importance of this TF region, anti-TF antibodies that bind to this region potently inhibit activation of substrates X and IX.

Another important interaction site with substrate is located in the VIIa protease domain centered around the inserted N-terminus. This exosite extends to the area of the Ca^{2+}-loop, a region that is topologically similar to the fibrinogen-binding exosite of thrombin. The crystal structure of the E76-

II. Screening of patient samples for expression of alt-hTF

SEQ ID NO:1 and SEQ ID NO:2 are useful in screening of patient samples for the presence of the normal alt-hTF protein, using hybridization assays of patient samples, including blood and tissues, as well as using the methods and reagents described in the examples. Screening can also be accomplished using antibodies, typically labeled with a fluorescent, radioactive, or enzymatic label, or by isolation of target cells and screening for clotting activity, as described in the examples. Typically, one would screen for expression on either a qualitative or quantitative basis, and for expression of functional alt-hTF.

Hybridization Probes

Reaction conditions for hybridization of an oligonucleotide probe or primer to a nucleic acid sequence vary from oligonucleotide to oligonucleotide, depending on factors such as oligonucleotide length, the number of G and C nucleotides, and the composition of the buffer utilized in the hybridization reaction. Moderately stringent hybridization conditions are generally understood by those skilled in the art as conditions approximately 25°C below the melting temperature of a perfectly base-paired double-stranded DNA. Higher specificity is generally achieved by employing incubation conditions having higher temperatures, in other words more stringent conditions. In general, the longer the sequence or higher the G and C content, the higher the temperature and/or salt concentration required. Chapter 11 of the well-known laboratory manual of Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, second edition, Cold Spring Harbor Laboratory Press, New York (1990), describes hybridization conditions for oligonucleotide probes and primers in great detail, including a description of the factors involved and the level of stringency necessary to guarantee hybridization with specificity.

The preferred size of a hybridization probe is from 10 nucleotides to 100,000 nucleotides in length. Below 10 nucleotides, hybridized systems are not stable and will begin to denature above 20°C. Above 100,000 nucleotides, one finds that hybridization (renaturation) becomes a much

To accomplish humanization of a selected mouse monoclonal antibody, the CDR grafting method described by Daugherty, et al., 1991 Nucl. Acids Res., 19:2471-2476, may be used. Briefly, the variable region DNA of a selected animal recombinant anti-idiotypic ScFv is sequenced by the method of Clackson, T., et al., 1991 Nature, 352:624-688. Using this sequence, animal CDRs are distinguished from animal framework regions (FR) based on locations of the CDRs in known sequences of animal variable genes. Kabat, H.A., et al., Sequences of Proteins of Immunological Interest, 4th Ed. (U.S. Dept. Health and Human Services, Bethesda, MD, 1987). Once the animal CDRs and FR are identified, the CDRs are grafted onto human heavy chain variable region framework by the use of synthetic oligonucleotides and polymerase chain reaction (PCR) recombination. Codons for the animal heavy chain CDRs, as well as the available human heavy chain variable region framework, are built in four (each 100 bases long) oligonucleotides. Using PCR, a grafted DNA sequence of 400 bases is formed that encodes for the recombinant animal CDR/human heavy chain FR protection.

The immunogenic stimulus presented by the monoclonal antibodies so produced may be further decreased by the use of Pharmacia's (Pharmacia LKB Biotechnology, Sweden) "Recombinant Phage Antibody System" (RPAS), which generates a single-chain Fv fragment (ScFv) that incorporates the complete antigen-binding domain of the antibody. In the RPAS, antibody variable heavy and light chain genes are separately amplified from the hybridoma mRNA and cloned into an expression vector. The heavy and light chain domains are co-expressed on the same polypeptide chain after joining with a short linker DNA that codes for a flexible peptide. This assembly generates a single-chain Fv fragment (ScFv) that incorporates the complete antigen-binding domain of the antibody. Compared to the intact monoclonal antibody, the recombinant ScFv includes a considerably lower number of epitopes, and thereby presents a much weaker immunogenic stimulus when injected into humans.

physically separated from the site of vascular injury. Recently, the role of blood-borne TF in thrombogenesis has been emphasized. In a rabbit venous thrombosis model, a specific anti-TF antibody inhibited thrombus formation. In this model, a collagen-coated cotton thread is inserted into the jugular vein and thrombus growth on the cotton-thread is monitored by measurement of the incorporated radioactive fibrinogen. Since no obvious vessel damage is involved in this model, it was concluded that the observed anti-thrombotic effect is due to inhibition of TF that is circulating in the blood. In parallel experiments, *ex vivo* perfusion of human blood over isolated pig aortic media and over collagen coated glass slides resulted in thrombi that stained for TF antigen. Inhibition of TF by a factor VIIa inhibitor reduced thrombus formation on the collagen coated slides. Furthermore, light- and electron-microscopic analysis of *ex vivo* human thrombi demonstrated TF staining of platelet surface, fibrin structures, monocytes and granulocytes. Because the samples were fixed with paraformaldehyde within five minutes, *de novo* synthesis of monocyte and granulocyte associated TF can be ruled out. Therefore, these experiments demonstrate the thrombogenic potential of blood-borne TF.

In a dog thrombosis model, granulocytes were associated with thrombi in damaged jugular veins and were shown to be the source of thrombus associated TF activity.

Elevated levels of shed membrane vesicles with procoagulant potential were detected in patients with acute coronary syndromes (Mallat, et al., Circulation 2000;101: 841-843; Kim, et al. Cardiology 2000;93: 31-36). Furthermore, procoagulant microparticles have been reported to be elevated in patients suffering meningococcal sepsis and patients with lupus anticoagulant (Nieuwland, et al., Blood 2000; 95; Combes, et al., J. Clin. Invest. 1999;104: 93-102). It is possible that circulating neutrophils might capture these vesicles and thereby serve as a carrier that provides the adhesion molecules necessary for recruitment of TF to growing thrombi.

A series of human studies also suggest that blood-borne TF might be an important factor in the etiology of several diseases. These studies

60). Early ideas about this correlation were focused on the TF-dependent initiation of proteolytic events leading to the formation of a fibrin coat on the surface of malignant cells that had entered the bloodstream after detachment from a primary tumor (Rickles and Edwards. Blood 1983;62: 14-31). This fibrin coat was thought to protect the circulating tumor cells from immune surveillance until they could adhere to the endothelium of a capillary bed and then become established as metastatic lesions (Hu, et al., Oncol. Res. 1994;6: 321-7). The correlation between metastatic potential and TF expression was brought into sharper focus when Mueller et al. (Murray, Br J Cancer 1991;64: 422-4) showed that highly metastatic human melanoma cells expressed levels of TF that were 1000 times higher than non-metastatic melanoma cells. The metastatic potential of the high TF expressing melanoma cell lines could be eliminated by incubation with an inhibitory monoclonal anti-TF antibody whereas incubation with a non-inhibitory monoclonal anti-TF antibody did not reduce the incidence of metastasis. These authors concluded that the procoagulant function of TF was essential for metastasis and that a downstream component of the coagulation cascade was responsible for the establishment of metastatic lesions. As work in this area progressed, reports began to emerge that linked TF to elevated levels of Vascular Endothelial Growth Factor (VEGF) and tumor angiogenesis (Abdulkadir et al. Hum Pathol 2000 31:443-447; Takano et al. Br J Cancer 2000 82:1967-1973).

In Clauss, et al., J. Exp. Med. 1990;172: 1535-45, a connection between TF levels, upregulation of VEGF, angiogenesis and tumor growth was demonstrated by transfection of a murine fibrosarcoma cell line (Meth-A) with mouse TF cDNA. The TF cDNA-transfected Meth-A sarcoma cell lines grew more rapidly than controls. The resulting tumors were more highly vascularized and produced increased amounts of VEGF mRNA compared to control cells that had been transfected with the same vector lacking the TF cDNA insert. These authors also reported that tumor cell mediated angiogenesis was independent of thrombin since hirudin did not inhibit the growth of endothelial cells treated with tumor cell supernatants.

metastasis in murine models. Various mechanisms, all involving active VIIa, have been proposed to account for these varied responses. Accordingly, one should be able to treat clotting or the disorders discussed above, by blocking the activity, or removal of, the alt-hTF, using standard techniques to obtain suitable antibodies or other compounds specific for the alt-hTF, or removal techniques such as a column or filter having immobilized therein on antibody specifically immunoreactive with the alt-hTF.

IV. Designing or screening for drugs modifying or altering the extent of alt-hTF function or expression

alt-hTF is useful as a target for compounds that turn on, or off, or otherwise regulate clotting or other disease processes mediated by alt-hTF. The assays described in the examples clearly provide routine methodology by which a compound can be tested. The *in vitro* studies of compounds that appear to inhibit alt-hTF are then confirmed by animal testing.

A number of animal models are useful and predictive of efficacy in humans. For example, as reviewed by Leadley, et al. J Pharmacol Toxicol Methods 2000 Mar-Apr;43(2):101-16, over the past two decades, great advances have been made in the pharmacological treatment and prevention of thrombotic disorders (e.g., tissue plasminogen activators, platelet GPIIb/IIIa antagonists, ADP antagonists such as clopidogrel, low-molecular weight heparins, and direct thrombin inhibitors). New research is leading to the next generation of antithrombotic compounds such as direct coagulation FVIIa inhibitors, tissue factor pathway inhibitors, gene therapy, and orally active direct thrombin inhibitors and coagulation Factor Xa (FXa) inhibitors. Animal models of thrombosis have played a crucial role in discovering and validating novel drug targets, selecting new agents for clinical evaluation, and providing dosing and safety information for clinical trials. In addition, these models have provided valuable information regarding the mechanisms of these new agents and the interactions between antithrombotic agents that work by different mechanisms. Genetic models have also been used in thrombosis/hemostasis research and pharmacology, for example, gene-

drug candidate, combinatorial chemists synthesize a series of closely related analogs. Computational chemistry tools are then used to simulate the interactions of structural elements with macro-molecules, such as receptors, in order to correlate structure with activity. Scientists need to be able to predict function based upon structural elements. Computational chemistry tools include tools for 3-D structure analysis, quantitative structure-activity relationship analysis, and comparative molecular field analysis, among others. Several companies market software and services to help speed drug discovery and lead optimization programs. For example, Tripos Inc., St. Louis, produces a variety of "chemically intelligent" modeling and analysis tools through its discovery software program. Bio Balance, New York, is an example of a company that does computer modeling of proteins for drug design.

Computer assisted drug design

Molecular modeling applications use falls into two broad categories: interactive visualization and computational analyses. The latter involves objective, computational analysis and is based upon known biophysical features of the molecule and established mathematical concepts that describe those features. These two approaches to modeling can be used alone or collectively to computationally derive a structure. Furthermore, these tools also can be used to reconstruct best-fit models from known structures when researchers make theoretical substitutions, insertions, or deletions in the composition of the macromolecule. Three of the most prominent uses of modern molecular modeling applications are structure analysis, homology modeling, and docking. Structure analysis centers on computational visualization of a molecule, provided its 3-D atomic coordinates have been elucidated, usually by X-ray crystallography or nuclear magnetic resonance (NMR). This information usually resides in major, world-accessible databases including the Brookhaven Protein Data Bank for protein structures, the Nucleic Acids DataBase at Rutgers University for DNA structures, and the Cambridge Crystallographic Data Centre (CCDC) for small molecule (nonprotein/DNA/RNA) structures. Using structure analysis tools,

Some of the more widely used docking programs include AutoDock (Oxford Molecular Group), DOCK (Molecular Design Institute-UC San Francisco), FTDOCK (Biomolecular Modeling Laboratory), INSIGHT II (MSI), SYBYL/FLEXIDOCK (Tripos) and MidasPlus (Computer Graphics Laboratory, UC San Francisco). Important challenges in this area are optimizing the conformations of the ligand and receptor, and modeling the relevant non-bonded interactions between two species. The docking programs GOLD (CCDC) and Flex X (Tripos) take the approach of applying data from X-ray crystal structures in the Cambridge Structural Database that is a source of experimental information on non-bonded contacts. Many commercial applications such as Cerius^2, INSIGHT II (MSI), HyperChem (Hypercube), Look /GeneMine (MAG), SYBYL (Tripos)—provide transparent interfaces to these tools. Structural images created from X-ray and NMR coordinate files represent a snapshot in time for any given structure. In reality, the atoms and molecules are constantly moving as a result of thermal molecular motion and interactions with their environment. This interaction represents both passive and active processes (for example, interactions with nearby water molecules and substrate, respectively). In either case, these interactions translate into structural change for the macromolecule. The molecular dynamics approach to structure analysis seeks to understand and predict these structural changes based upon energy minimization. Dynamics analyses are based on an assessment (usually performed by molecular mechanics methods) of the free energy changes between two different structural states (a protein with and without bound ligand, for example). By mathematically extrapolating free energy changes, one can model a particular structure, which would have the appropriate calculated total free energy. These calculations can be reiterated for practically an infinite set of time points, thus allowing a researcher to model the temporal dynamics of macromolecule structure, for instance, as it performs some catalytic or binding function. Modeling programs that utilize molecular dynamics function include HyperChem (Hypercube), INSIGHT II/Discover (MSI), AMBER, CAChe (OMG), SYBYL (Tripos), Alchemy

(Computer Graphics Lab), and SYBYL (Tripos) are especially important to researchers performing homology modeling and docking, where various kinds of computational routines are utilized in the model-building process. Such a process incorporates all physicochemical properties into the computational equation to derive the best thermodynamically stable structure, a structure that should depict a functional molecule.

Computer modeling technology allows visualization of the three-dimensional atomic structure of a selected molecule and the rational design of new compounds that will interact with the molecule. The three-dimensional construct typically depends on data from x-ray crystallographic analyses or NMR imaging of the selected molecule. The molecular dynamics require force field data. The computer graphics systems enable prediction of how a new compound will link to the target molecule and allow experimental manipulation of the structures of the compound and target molecule to perfect binding specificity. Prediction of what the molecule-compound interaction will be when small changes are made in one or both requires molecular mechanics software and computationally intensive computers, usually coupled with user-friendly, menu-driven interfaces between the molecular design program and the user.

Although described above with reference to design and generation of compounds that could alter binding, one could also screen libraries of known compounds, including natural products or synthetic chemicals, and biologically active materials, including proteins, for compounds that are useful.

Generation of nucleic acid regulators

Nucleic acid molecules containing the 5' regulatory sequences of the TF gene can be used to regulate or inhibit gene expression *in vivo*. Vectors, including both plasmid and eukaryotic viral vectors, may be used to express a particular recombinant 5' flanking region-gene construct in cells depending on the preference and judgment of the skilled practitioner (see, e.g., Sambrook et al., Chapter 16). Furthermore, a number of viral and nonviral vectors have been developed that enable the introduction of nucleic acid

1993 FASEB J. 7, 533-539.) Inhibition of expression of a gene by antisense oligonucleotides is also possible if the antisense oligonucleotides contain modified nucleotides (see, e.g., Offensperger et. al., 1993 EMBO J. 12, 1257-1262 (antisense phosphorothioate oligodeoxynucleotides); Rosenberg et al., PCT WO 93/01286 (synthesis of sulfurthioate oligonucleotides); Agrawal et al., 1988 Proc. Natl. Acad. Sci. USA 85, 7079-7083 (synthesis of antisense oligonucleoside phosphoramidates and phosphorothioates); Sarin et al., 1989 Proc. Natl. Acad. Sci. USA 85, 7448-7794 (synthesis of antisense methylphosphonate oligonucleotides); Shaw et al., 1991 Nucleic Acids Res 19, 747-750 (synthesis of 3' exonuclease-resistant oligonucleotides containing 3' terminal phosphoroamidate modifications).

Inhibition of expression of a gene can also be achieved by using small double-stranded RNA molecules (21-25 base pairs in length) by means of a process known as RNA interference (see, e.g., Sharp, Genes & Development, 2001, 15:485-490). Double-stranded RNA molecules can be synthesized *in vitro* and then introduced into living cells (see, e.g., Donze et al., 2002 Nucleic Acid Research, 30:e46) or synthesized from a DNA template that was stably incorporated into cells (see, e.g., Sui et al., 2002 Proc. Natl. Acad. Sci. USA 99:5515-5520). Double-stranded RNA molecules have been shown to inhibit HIV-1 infection (see Novina et al., 2002, Nature Medicine, 8:681-686) and expression of the full-length tissue factor (see Holen et al., Nucleic Acid Research 2002, 30:1757-1766). Thus, double-stranded RNA molecules containing the region unique to alt-hTF mRNA, i.e. the site of splicing of exon 4 to exon 6, may be used to selectively inhibit expression of alt-hTF protein in vivo.

The sequences of the 5' flanking region of the TF gene can also be used in triple helix (triplex) gene therapy. Oligonucleotides complementary to gene promoter sequences on one of the strands of the DNA have been shown to bind promoter and regulatory sequences to form local triple nucleic acid helices which block transcription of the gene (see, e.g., 1989 Maher et al., Science 245, 725-730; Orson et al., 1991 Nucl. Acids Res. 19, 3435-3441; Postal et al., 1991 Proc. Natl. Acad. Sci. USA 88, 8227-8231; Cooney

helices specifically within the 5' flanking region of the TF gene in order to inhibit expression of the gene.

In some cases it may be advantageous to insert enhancers or multiple copies of the regulatory sequences into an expression system to facilitate screening of methods and reagents for manipulation of expression.

Random generation of alt-hTF encoding sequence binding molecules

Molecules with a given function, catalytic or ligand-binding, can be selected for from a complex mixture of random molecules in what has been referred to as "in vitro genetics" (Szostak, TIBS 19:89, 1992). One synthesizes a large pool of molecules bearing random and defined sequences and subjects that complex mixture, for example, approximately 10^{15} individual sequences in 100 μg of a 100 nucleotide RNA, to some selection and enrichment process. For example, by repeated cycles of affinity chromatography and PCR amplification of the molecules bound to the ligand on the column, Ellington and Szostak (1990) estimated that 1 in 10^{10} RNA molecules folded in such a way as to bind a given ligand. DNA molecules with such ligand-binding behavior have been isolated (Ellington and Szostak, 1992; Bock et al, 1992).

Preparation of alt-hTF protein fragments

Compounds that are effective for blocking binding of the alt-hTF can also consist of fragments of the alt-hTF protein, expressed recombinantly and cleaved by enzymatic digest or expressed from a sequence encoding a peptide of less than the full length alt-hTF protein. These will typically be soluble proteins, i.e., not including the transmembrane and cytoplasmic regions, although smaller portions determined in the assays described above to inhibit or compete for binding to the alt-hTF protein can also be utilized. It is a routine matter to make appropriate alt-hTF protein fragments, test for binding, and then utilize them. The preferred fragments are of human origin, in order to minimize potential immunological response. The peptides can be as short as five to eight amino acids in length and are easily prepared by standard techniques. They can also be modified to increase *in vivo* half-life,

Peptides containing cyclopropyl amino acids, or amino acids derivatized in a similar fashion, can also be used. These peptides retain their original activity but have increased half-lives in vivo. Methods known for modifying amino acids, and their use, are known to those skilled in the art, for example, as described in U.S. Patent No. 4,629,784 to Stammer.

V. Pharmaceutical compositions

Peptides are generally active when administered parenterally in amounts above about 1 μg/kg of body weight. Based on extrapolation from other proteins, for treatment of most inflammatory disorders, the dosage range will be between 0.1 to 70 mg/kg of body weight. This dosage will be dependent, in part, on whether one or more peptides are administered. In the preferred embodiment, with regard to alt-hTF, the peptide sequences present in the C-terminal region of alt-hTF (amino acid residues 167-206) should be the most optimal. Because this sequence has not been described in other proteins, this would be a unique target to inhibit binding alt-hTF to platelets, thereby uniquely inhibiting the activity of alt-hTF.

Compounds that alter alt-hTF protein activity and/or binding (referred to generally herein as "binding activity") are preferably administered in a pharmaceutically acceptable vehicle. Suitable pharmaceutical vehicles are known to those skilled in the art. For parenteral administration, the compound will usually be dissolved or suspended in sterile water or saline. For enteral administration, the compound will be incorporated into an inert carrier in tablet, liquid, or capsular form. Suitable carriers may be starches or sugars and include lubricants, flavorings, binders, and other materials of the same nature. The compounds can also be administered locally by topical application of a solution, cream, gel, or polymeric material (for example, Pluronic™, BASF).

Alternatively, the compound may be administered in liposomes or microspheres (or microparticles). Methods for preparing liposomes and microspheres for administration to a patient are known to those skilled in the art. U.S. Patent No. 4,789,734 describes methods for encapsulating biological materials in liposomes. Essentially, the material is dissolved in an

layers were evident and their volume fractions were estimated: an upper lipid phase (17% of volume), a bulk-aqueous phase (52%), a lower viscous phase (23%) and a pellet (3%). Samples from the starting material and each of the three upper phases were extracted and assayed for TF activity using a standardized protocol. In one experiment, the pellet was resuspended in 4.5mL of TBS and also assayed for TF activity.

In the standardized assay for TF activity, 480 μL of a given sample is incubated with 20 μL of Triton X-100 for 1 hour, and then incubated overnight with 65 μL of agrose beads covalently linked to a rabbit anti-human TF antibody (0.5 mg antibody per mL of agarose). The beads were washed 5 times with hepes-buffered saline (HBS) and the bound antigen was eluted with 6M guanidine dissolved in HBS. The eluted protein was collected and incubated with phospholipids (30% phosphatidyl serine, 70% phosphatidyl choline; 75 μM total) and n-octyl-β-D-glucopyranoside. The samples were dialyzed to remove the detergent and allow the formation of lipid vesicles in the presence of the eluted protein. The dialyzed samples were supplemented with calcium (5mM), coagulation factor VIIa (1 nM) and coagulation factor X (150 nM), and the rate of appearance of FXa was measured using a standard assay.

The results are shown in Figure 2. The TF activity associated with the starting material was 158.5 pM-FXa/min. Following ultracentrifugation, TF activity measured in each phase was 45.7 pM-FXa/min for the lipid phase, 138.9 pM-FXa/min for the bulk aqueous phase, 554.7 pM-FXa/min for the lower viscous phase, and 40 pM-FXa/min for the resuspended pellet. Background levels were approximately 20 pM-FXa/min.

In a separate experiment, the immuno-capture procedure was omitted and plasma samples were relipidated directly with phospholipid (75μM) and n-octyl-β-D-glucopyranoside. The detergent was dialyzed out and TF activity was measured as described above. There was no increase in the concentration of FXa with time, indicating that the TF present was either in an inactive form or inhibited.

terminus diverge from the known TF amino acid sequence. Of note, the carboxy-terminus of the alternatively spliced hTF contains a region with potential transmembrane properties.

Example 3: Expression of alt-hTF in various tissues.

In order to determine whether the alternatively spliced hTF mRNA is expressed in various tissues, a BLAST search of EST libraries was conducted for the presence of clones that contain the site of alternative splicing, i.e. the end of exon 4 fused directly with start of exon 6. Four EST clones have been identified, and their sequences were analyzed for the presence of open reading frames using the Translate tool available at http://www.expasy.ch/tools/dna.html.

The lengths of reported sequences of the three clones derived from a human lung cDNA library (BG506479, BG539133, and BG546020) are 663, 780, and 747 base pairs, respectively. None of the three sequences encode a significantly larger non-interrupted stretch of amino acids in any of the three 5'-3' frames. Although all three sequences contain a region corresponding to the site of alternative splicing, i.e., TCA GGA AAG AAA TAT TCT (SGKKYS) (SEQ ID NO:3), in all three sequences this region is not in frame with the sequence encoding human tissue factor protein. The three clones corresponding to the above lung ESTs were obtained from the I.M.A.G.E. consortium and fully sequenced. None of the clones contained the complete alt-hTF open reading frame. However, sequencing results revealed that, like in membrane-bound TF mRNA, the long 3'-untranslated end of the asHTF mRNA is entirely encoded by exon 6.

The length of the reported sequence of a clone derived from a primary human keratinocyte cDNA library (BF149254) is 356 base pairs. This sequence encodes a stretch of amino acids 72 through 166 of the membrane bound human tissue factor fused to the first 23 amino acids of the 40 amino acid carboxyl-terminus unique to the alternatively spliced hTF. This sequence is, therefore, a partial (incomplete) cDNA encoding an alternatively spliced human tissue factor molecule.

FVIIai inhibited this activity by about 60%. The flow-through sample, upon incubation with platelets as well as shear, generated insignificant amounts of Xa (~0.8 nM).

Example 5: Expression of the alt-hTF protein in bacteria.

The region encoding the entire mature peptide of the alternatively spliced hTF variant was amplified using RT-PCR and subcloned into pBAD/gIIIA expression vector (Invitrogen Corporation). The sequence of this construct was verified, and the recombinant alternatively spliced hTF protein was produced in *E. coli*. At the N-terminus of the expressed protein, three additional amino acids (i.e. Thr, Met, and Ala) were present due to the structure of the multiple cloning site in pBAD/gIIIA expression vector. The expressed protein was isolated from bacteria by osmotic shock, and the resultant osmotic shock fluid was concentrated via centrifugation in Centricon™ filter devices (Millipore). Presence of the desired protein in the concentrated osmotic shock fluid was verified by Western immunoblotting, and protein concentration was analysed using Bradford protein microassay.

Three samples containing the recombinant alt-hTF protein were then prepared: a sample of the protein dissolved in HBS, a sample of the relipidated protein, and a sample of the protein incubated with phospholipid vesicles. Relipidation of the recombinant protein was carried out as follows. 35 μg of protein in TBS were combined with n-octyl-β-D-glucopyranoside (final concentration – 125 mM) and a phospholipid mixture (PS:PC 30:70, final concentration – 75 μM) to the final volume of 0.5 mL. The sample was placed on orbital mixer (set to slow) for 30 min at RT, transferred into a 0.5 mL dialysis cassette and dialyzed overnight versus 2 L of TBS. Incubation of the recombinant protein with phospholipid vesicles was carried out as follows. 35 μg of protein in HBS were combined with a phospholipid vesicle preparation (PS:PC 30:70, final concentration – 75 μM) that was extruded to yield vesicles with the diameter of 100 nm. This mixture was incubated overnight at room temperature.

The activity of relipidated alt-hTF, alt-hTF incubated with phospholipid vesicles, and plain alt-hTF was measured in the following

samples were added to the well of a 96 well plate containing EDTA (final concentration = 25 mM). A chromogenic Xa substrate, Spectrozyme Xa, was then added to the well (final concentration = 0.5 mM), and the plate was read for 15 minutes at 405 nM.

The results of this assay are shown in Figure 4B. A suspension of isolated platelets, platelets mixed with the relipidated alt-hTF protein, as well as the polyclonal antibody to this protein and the platelet-alt-hTF suspension treated with FVIIai exhibited no Xa generation. When relipidated alt-hTF was mixed with platelets and sheared, a significant amount of Xa (~5 nM) was generated.

Example 6: Presence of the alt-TF protein in *ex-vivo* thrombi.

To determine which forms of TF are incorporated into *ex-vivo* thrombi, immunohistochemistry was performed on thrombi formed by shearing whole human blood over collagen-coated cover slips (650 s^{-1}). These thrombi stained with antibodies against alt-TF and sTF but stained only marginally, if at all, with an antibody specific for full-length TF, indicating that alt-hTF was selectively incorporated into thrombi formed under these conditions.

16. The method of claim 15 wherein the levels are determined using an immunoassay with an antibody specifically reactive with alternatively spliced tissue factor.

17. An inhibitor of the tissue factor proteins of any of claims 1-8 that is not reactive with membrane bound human tissue factor.

18. A method of treating a patient with a disorder associated with tissue factor comprising administering an effective amount of the inhibitor of claim

19. An antibody specifically immunoreactive with the tissue factor proteins of any of claims 1-8.

20. The antibody of claim 19 conjugated to a detectable label.

21. The antibody of claim 19 immobilized to a column or filter.

22. A method of treating a patient with a disorder associated with tissue factor comprising removing alternatively spliced tissue factor using the antibody of claim 19.

Figure 2

TF activity for the fractions of human plasma centrifuged at 260,000xG

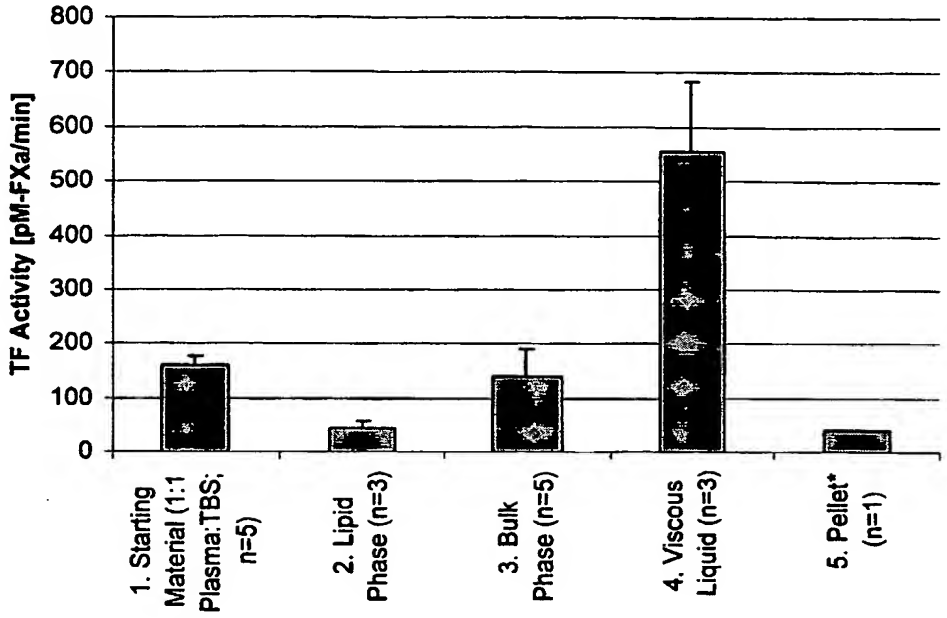
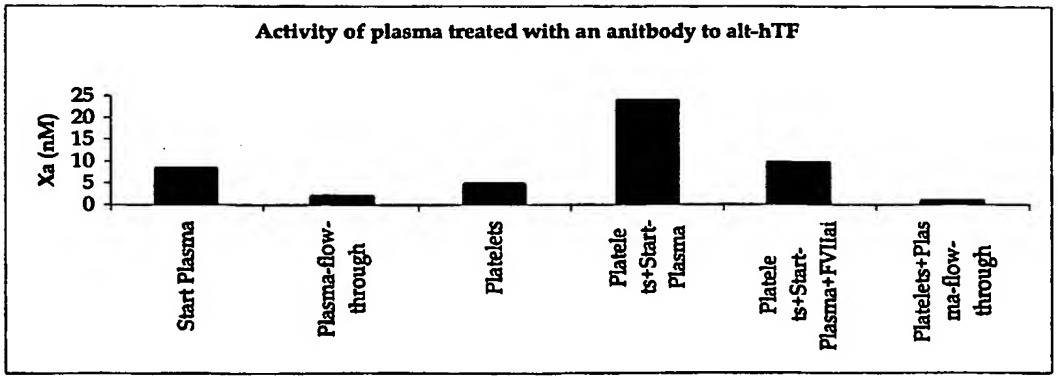


Figure 3



SEQUENCE LISTING

<110> Mount Sinai School of Medicine of New York University

<120> ALTERNATIVELY SPLICED CIRCULATING TISSUE FACTOR

<130> MSM 102 PCT

<150> US 60/316,244
<151> 2001-08-30

<160> 3

<170> PatentIn version 3.1

<210> 1
<211> 40
<212> PRT
<213> Homo sapiens

<400> 1

Tyr Ser Thr Ser Leu Glu Leu Trp Tyr Leu Trp Ser Ser Ser Leu Ser
1 5 10 15

Ser Ser Trp Leu Tyr Leu Tyr Thr Ser Val Glu Arg Gln Glu Trp Gly
20 25 30

Arg Ala Gly Arg Arg Thr Pro His
35 40

<210> 2
<211> 123
<212> DNA
<213> Homo sapiens

<400> 2
tattctacat cattggagct gtggtatttg tggtcatcat ccttgtcatc atcctggcta 60

tatctctaca caagtgtaga aaggcaggag tggggcagag ctggaaggag aactccccac 120

tga 123

<210> 3
<211> 24
<212> DNA
<213> Homo sapiens

<400> 3
tcaggaaaga aatattctsg kkys 24

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 March 2003 (13.03.2003)

PCT

(10) International Publication Number
WO 2003/020111 A3

(51) International Patent Classification[7]: C12P 21/06, C07K 14/00, C07H 17/00

(21) International Application Number: PCT/US2002/027533

(22) International Filing Date: 29 August 2002 (29.08.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/316,244 30 August 2001 (30.08.2001) US

(71) Applicant: **MOUNT SINAI SCHOOL OF MEDICINE OF NEW YORK UNIVERSITY** [US/US]; One Gustave L. Levy Place, New York, NY 10029-6574 (US).

(72) Inventors: **NEMERSON, Yale**; 145 Nassau Street, Apartment 10C, New York, NY 10038 (US). **BOGDANOV, Vladimir**; 508 East 79th Street, Apartment 6A, New York, NY 10021 (US).

(74) Agents: **PABST, Patrea, L.** et al.; Holland & Knight LLP, One Atlantic Center, 1201 West Peachtree Street, N.E., Suite 2000, Atlanta, GA 30309-3400 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
29 January 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ALTERNATIVELY SPLICED CIRCULATING TISSUE FACTOR

(57) Abstract: A new circulating foml of soluble human tissue factor was identified. This new foml of human tissue factor appears to be the result of alternative splicing and is therefore referred to as "alt-hTF." Alt-hTF mRNA was detected in a cell line, HL-60. The cDNA region encoding the entire open reading frame of alt-hTF was cloned. The sequence encoding the alt-hTF mature peptide was expressed in bacteria. alt-hTF consists of the first 166 amino acids of membrane bound TF, and a 40 amino acid C-temlinal region unique to alt-hTF. Alt-hTF is likely to be a useful target for compounds to inhibit clotting and to treat disorders associated with elevated TF. It may also be useful as a target for antibodies selectively reactive with alt-hTF, to remove it from the circulation for treatment of clotting or other disorders associated with elevated or abnomlallevels of TF, including thrombotic conditions, cardiovascular disorders, DVT, DIC, and possibly metastatic cancers.

WO 2003/020111 A3